CHARACTERIZATION OF MILK CORTISOL CONCENTRATIONS AS A MEASURE OF SHORT-TERM STRESS RESPONSES IN LACTATING DAIRY COWS

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This study compares cortisol concentrations in plasma and milk over a time-span of 1-4h following injection of an adrenocorticotropic hormone (ACTH) or a physiological stressor. Its aim was to characterize the usefulness of milk cortisol concentrations as short-term measures of acute stress.

Experiment 1: three groups of lactating cows (Groups B – D; n = 5, each) were injected with $ACTH_{1-24}$ at 4, 2 and 1h before milking, respectively, so that each experienced a similar period of elevated adrenocortical activity, but presented at milking with varying plasma cortisol concentrations. Another group (Group A; n = 5) was a saline-treated control. Mean plasma cortisol concentrations at milking were 7, 8, 24 and 56ng ml^{-1} for Groups A, B, C and D, respectively. Mean cortisol concentrations in milk were similar for Groups A and B (1.2 and 0.5 ng ml^{-1}, respectively), higher in Group C (2.4ng ml^{-1}), and greatest in Group D (11.7 ng ml^{-1}; $P < 0.001$).

Experiment 2: lactating dairy cows (n = 15) were injected with ACTH, transported by truck, or blood-sampled only (control) during the 2h before milking. Mean plasma cortisol concentrations at milking were 6, 20 and 72 ng ml^{-1} following control, ACTH and transport treatments, respectively; mean concentrations of cortisol in milk displayed a similar pattern (1.1, 2.4 and 12.0 ng ml^{-1}, respectively; $P < 0.001$).

Milk cortisol concentrations were highly correlated with plasma levels at milking, but did not reflect those situations where, following a period of elevation within the previous 4h, plasma cortisol concentrations had returned to basal levels. Concentrations of cortisol in foremilk and composite milk were highly correlated, but the mechanisms of cortisol flux may

differ between these two compartments. Milk cortisol concentration can be a useful indicator of responses of lactating cows to acute stressors which act up to 2h before collection of samples.

Keywords: *ACTH, animal welfare, cortisol, dairy cow, milk, plasma, stress, transport*

Introduction

An increase in the concentration of cortisol in plasma is indicative of an adrenocortical response to an acute stressor (Tarrant *et al* 1992). Unfortunately, there are few non-stressful blood sampling techniques available for free-ranging animals and results are frequently confounded by the procedures required for sample collection (Ingram *et al* 1994; Carragher *et al* 1997). Measurements of cortisol concentrations in other biological fluids, eg faeces and urine, have provided useful information without disturbing the animal (Miller *et al* 1991). For dairy cows, concentrations of cortisol in milk may be a useful indicator of adrenocortical activity, since milk collection is a routine and regular operation creating minimal disturbance to the animal. Changes in the concentration of cortisol in milk have been used to measure responses of the hypothalamo-pituitary-adrenal (HPA) axis of lactating animals to stress associated with transport and a novel farm environment (Bremel and Gangwer 1978).

A number of workers have investigated the dynamics of the relationship between plasma and milk cortisol concentrations using adrenocorticotropic hormone (ACTH) and/or cortisol injections (Gwazdauskas *et al* 1977; Bremel and Gangwer 1978; Fox *et al* 1981; Shutt and Fell 1985). Their results suggest that concentrations of cortisol in plasma and milk are in equilibrium, and that free cortisol moves readily between compartments by simple diffusion. Most of these experiments have utilized pharmacological doses of ACTH (100 - 250iu) and broad time-courses (between 4h and 6 days) and are of limited value for interpretation of milk cortisol concentrations when lactating cows are exposed to physiological stressors within a shorter term.

The aim of the present study was to assess the relative changes in cortisol concentrations in plasma and milk following activation of the HPA axis for up to 2h with a recently developed low-dose ACTH challenge (Verkerk *et al* 1994), or a physiological stressor (transport). The relationship between cortisol concentrations in samples of milk stripped from the udder before milking (foremilk) and in composite milk (milk collected by machine milking after milk let-down) was also examined.

Materials and Methods

All procedures were approved by the Animal Ethics Committee, AgResearch, Hamilton, New Zealand.

Experiment 1:

Animals

Twenty multiparous dairy cows, aged between 3 and 8 years (Holstein-Friesian, n = 11; Jersey, n = 9) and in mid-lactation, were randomly assigned to one of four groups (n = 5 each). The experiments were conducted during summer (November) 1995. All cows were within the first trimester of pregnancy at the time of the experiment. Cows were grazed on

ryegrass and clover pasture in close proximity to the handling area at Ruakura, Hamilton, New Zealand, to which they were moved quietly for blood sampling at the appropriate times.

Treatments
Group A received a saline injection at 1100h, and Groups B, C and D were administered synthetic ACTH at 1100h, 1300h or 1400h respectively. The ACTH treatment was based on an adrenal response test previously validated for use in cattle (Verkerk *et al* 1994). A dose of 0.05mg of $ACTH_{1-24}$ (40iu; Synacthen™, CIBA-Geigy, Switzerland) was prepared, in a total volume of 2ml physiological saline, and injected into the coccygeal vein of each animal.

Sampling
Blood samples were collected into heparinised vacutainers by coccygeal venipuncture. Saline-treated cows (Group A) were sampled at 1100h, 1300h and 1500h. Cows in Groups B, C and D were sampled immediately before the ACTH injection, and again after 60min (expected peak plasma cortisol concentration). Additional samples were taken from cows in Groups B and C at 120min after ACTH administration (before milking for Group C). A fourth sample was taken before milking from cows in Group B. Immediately after the final blood sample was taken, cows moved to the milking platform (maximum time between blood and milk sampling 10min). Total milk weights were estimated and composite milk samples were collected using a proportioning milk meter (Tru Test Co, Auckland, New Zealand) at each milking on the day of treatment (morning milking: approximately 0700h; afternoon milking approximately 1500h). Foremilk samples (approximately 2.5ml of milk removed from each quarter of the udder by hand prior to machine milking) were collected immediately before the afternoon milking.

Experiment 2:
Animals
Fifteen multiparous dairy cows, aged between 3 and 5 years (Holstein-Friesian, n = 8; Jersey, n = 7) and in mid-lactation, were randomly assigned to three groups (n = 5 each). The cows were managed as for Experiment 1.

Treatments
Three treatments (control, ACTH and transport) were administered in a 3x3 Latin square design with at least 72h between treatments. The only procedure used for the control treatment was blood sampling. The ACTH treatment, as described in Experiment 1, commenced at 1300h. Cows allocated to the transport treatment were loaded onto a truck (at a density of $1.0m^2$ per cow) following collection of a blood sample at 1300h. The truck was driven over a designated route for 45min, then the cows were unloaded. A further blood sample was collected before the loading, transport and sampling were repeated.

Sampling
Total milk weights were estimated and composite samples were collected at both milkings on each treatment day. All cows sampled hourly from 1300h to 1500h had their blood and then their milk sampled within 10min of the third blood sampling.

Sample handling and assays

Blood samples were stored in iced water, and centrifuged (1500g for 15min) within 60min of collection. Plasma was aspirated and stored at -20°C until analysed. After mixing, a 10ml portion of foremilk and composite milk samples were de-fatted by centrifugation (1500g for 15min), and the skimmed milk fractions stored at -20°C until analysed. A further 30ml sub-sample of each composite milk sample was submitted for infrared analysis of its fat, protein and lactose content (Milkoscan 133B, Foss Electric, Denmark), and for assessment of the somatic cell count (Fossomatic, Foss Electric, Denmark).

Total cortisol concentrations in milk and plasma samples were measured in duplicate using an ^{125}I radio-immunoassay with polyethylene glycol separation following extraction with ethyl acetate as previously described by Ingram *et al* (1994). Plasma and milk cortisol concentrations were measured in separate assays. The mean intra-assay and inter-assay coefficients of variation were 9.5 and 15.2 per cent, respectively. The minimum detection limit was 0.25 ng ml^{-1}.

Statistical analyses

Results are reported as means ± SEMs. The total mass of fat, protein and lactose at each milking were calculated from milk weight and composition data. Total milk cortisol for each cow at the afternoon milking was calculated using milk weights and composite milk cortisol concentrations. In Experiment 1, the area under the plasma cortisol concentration vs time curve for each cow over the period from 1100h to 1500h was calculated to measure the integrated adrenocortical response (Verkerk *et al* 1994). The ratios of cortisol concentrations in milk and plasma were calculated for each cow at each treatment.

Data from both experiments were examined by analyses of variance (ANOVA) using a general linear model (GLM) with Minitab (version 8.2; Minitab Statistical Software, State College, Pennsylvania, USA) for effects associated with treatment (Experiment 1) and with treatment, cow, and day of treatment (Experiment 2). Analyses of milk weight and composition included values from the morning milking as covariates to correct for individual cow variation. Cortisol concentrations in foremilk and composite milk samples from Experiment 1 were compared using a paired Student's *t* test. Linear regression analyses, using Minitab, tested for relationships between cortisol concentrations in milk and plasma in both experiments. Plasma cortisol concentrations at 60min following ACTH treatment and after 60min of transport (Experiment 2) were further examined to assess the consistency of the cows' responses. Repeatability of response was calculated by partitioning the variance into the significant components of cow, day and error (repeatability = $(\sigma^2_{cow}) / (\sigma^2_{cow} + \sigma^2_{day} + \sigma^2_{error})$; Falconer (1960). Cows were ranked according to response for each treatment and rank correlations calculated.

Results

Experiment 1

Milk cortisol concentrations in composite milk samples collected at the morning milking were similar for all treatment groups (overall mean 0.8 ± 0.1ng ml^{-1}). Levels in control cows were greater at the afternoon milking than at the morning milking (1.2 ± 0.2 and 0.8 ± 0.1 ng ml^{-1}, respectively, $P < 0.05$). Plasma cortisol concentrations at the start of treatments were low, with significant variation between groups ($P < 0.05$) such that levels

in Groups A and B (both 11.3±2.4ng ml^{-1}) were higher than in Groups C and D (4.7 ± 1.3 and 3.6 ± 0.4 ng ml^{-1}, respectively). There was no significant change in plasma cortisol concentrations following saline treatment. Injection of ACTH produced similar elevations of plasma cortisol concentration at 60min in groups B, C and D (overall mean 58.5 ± 6.2ng ml^{-1}); but the mean integrated adrenocortical response was greater in Group B than in Groups C and D ($P < 0.05$; Table 1). The treatments resulted in a range of plasma cortisol concentrations immediately before the afternoon milking, such that levels were similar in Groups A and B, significantly higher in Group C, and highest in Group D ($P < 0.001$; Table 1). Cortisol concentrations in foremilk and composite samples, and total milk cortisol are given in Table 1. These variables were similar between groups A and B, significantly higher in group C, and greatest in Group D ($P < 0.001$).

Table 1 **Mean (and SEM) of plasma cortisol concentrations at time of afternoon milking, integrated adrenocortical responses, cortisol concentrations in foremilk and composite milk samples, and total milk cortisol in post-treatment samples at afternoon milking in Experiment 1. (Different superscript letters represent significant differences within columns: $P < 0.05$ for area, and $P < 0.001$ for plasma and milk cortisol variables.)**

Treatment Group	Plasma cortisol[1] (milking)	Area[2]	Foremilk cortisol[1]	Composite milk cortisol[1]	Total milk cortisol[3]
A: Control	7.1 (1.9)^a	2634 (445)^a	0.7 (0.1)^a	1.2 (0.2)^a	8 (2)^a
B: ACTH (1100h)	8.3 (2.3)^a	5096 (834)^b	0.8 (0.1)^a	0.5 (0.1)^a	3 (1)^a
C: ACTH (1300h)	23.9 (5.1)^b	3679 (421)^c	5.8 (0.8)^b	2.4 (0.8)^b	13 (4)^b
D: ACTH (1400h)	56.0 (6.5)^c	4214 (431)^c	9.8 (1.5)^c	11.7 (2.4)^c	81 (16)^c

[1] ng ml^{-1}

[2] areas under the plasma cortisol concentration: time curve from 1100h to 1500h; ng min ml^{-1}

[3] μg

Table 1 shows that concentrations of cortisol in foremilk and composite milk samples were highly correlated ($r = 0.92$). They were similar within treatment groups, except in Group C, in which the mean cortisol concentration was higher in foremilk than in composite milk ($P < 0.01$). The mean ratio of these concentrations varied between treatments such that they were similar for Groups A and D (0.7 ± 0.2 and 0.9 ± 0.1ng ml^{-1}, respectively) and Groups B and C (1.6 ± 0.2 and 2.5 ± 0.5 ng ml^{-1}, respectively) although the means for groups A and D differed from those for groups B and C ($P < 0.01$).

The best-fit regression relationships between concentrations of cortisol in plasma at the time of milking and the two types of milk sample were linear, with similar slopes (Figure 1). Although the regression coefficients for these relationships were both high ($r = 0.70$, $P < 0.001$ for composite milk and $r = 0.71$, $P < 0.001$ for foremilk), residual plots indicated that cortisol concentrations in composite milk samples more accurately reflected plasma cortisol concentrations over the range of values examined. The linear model did not fully explain cortisol concentrations in foremilk samples when plasma cortisol concentrations were at either end of the range.

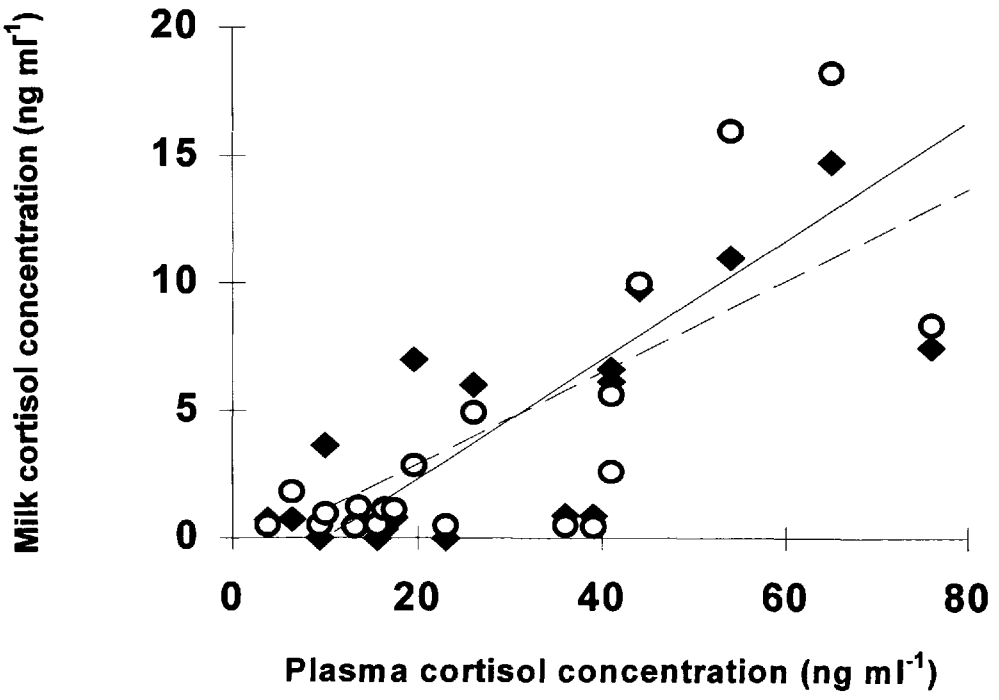

Figure 1 **Relationship between total cortisol concentration in foremilk or composite milk and that of plasma samples taken immediately before milking.**

Legend:
- o - **foremilk samples**
- ◆ - **composite milk samples**
- - - - - **regression line for foremilk samples**
- ____ - **regression line for composite milk samples**

Milk weights and somatic cell counts at the afternoon milking did not vary between treatment groups (mean somatic cell count 41 ± 6 x 10^3 cells ml^{-1}); nor were there differences between treatment groups for any milk composition variable, although data from the morning milking were significant covariates for each variable studied, respectively (data not shown).

Experiment 2

Milk cortisol concentrations at the morning milking were similar for all treatment groups (overall mean 0.7 ± 0.1 ng ml^{-1}), but less than those following the control treatment at the afternoon milking (1.1 ± 0.1 ng ml^{-1}; $P < 0.02$). Initial plasma cortisol concentrations (at 0min of treatment) were similar for all groups (overall mean 9.9 ± 1.0 ng ml^{-1}).

Mean values for plasma cortisol concentrations at 60 and 120min after the start of treatment are given in Table 2. Plasma cortisol concentrations remained at basal levels during the sampling period in control cows. At 60min following the start of treatments, plasma cortisol concentrations were elevated to a similar degree in the ACTH and transport treatments and were greater ($P < 0.001$) than in the control cows. By 120min following the start of treatment, levels had decreased with the ACTH treatment, but were still higher ($P < 0.001$) than controls, whereas levels with the transport treatment were similar to those seen at 60min.

Table 2 **Mean (and SEM) cortisol concentrations of plasma and milk, milk weights and total milk cortisol in post-treatment samples at afternoon milking in Experiment 2. (Different superscript letters represent significant differences within columns: $P < 0.001$ for plasma and milk cortisol variables and $P < 0.05$ for milk weight.)**

Treatment Group	Plasma cortisol (at 60min)[1]	Plasma cortisol (at 120min)[1]	Milk cortisol[1]	Milk weight[2]	Total milk cortisol[3]
Control	6.6 (0.9) [a]	6.4 (0.9) [a]	1.1 (0.1) [a]	5.7 (0.2) [a]	6 (1)[a]
ACTH	63.7 (2.9) [b]	19.8 (2.3) [b]	2.4 (0.4) [b]	5.9 (0.4) [a]	14 (2)[b]
Transport	65.9 (3.7) [b]	72.4 (2.9) [c]	12.0 (1.2) [c]	4.4 (0.5) [b]	53 (9)[c]

[1] ng ml^{-1}
[2] kg
[3] μg

Initial plasma cortisol concentrations were correlated ($P < 0.05$) with those at 60min (r = 0.69 and 0.59 for ACTH and transport treatments, respectively). When data for initial plasma cortisol concentrations were included in the GLM ANOVA as a covariate, there were significant effects of both cow and day ($P < 0.02$ and $P < 0.03$, respectively). The repeatability of individual cow response was 34 per cent. There was a significant rank correlation between responses to each treatment (r = 0.54, $P < 0.05$).

Concentrations of cortisol in milk samples collected at the afternoon milking were significantly different between each treatment ($P < 0.001$), being greatest following transportation (Table 2). Milk cortisol concentrations at the afternoon milking showed a linear relationship with plasma cortisol concentrations immediately before milking (r = 0.80, $P < 0.001$). Mean milk weight was less ($P < 0.05$) following the transport treatment than either control or ACTH treatments, which were similar (Table 2). Total milk cortisol differed ($P < 0.001$) between each treatment in the same manner as milk cortisol concentrations (Table 2). Treatment did not significantly influence any milk composition variable examined (data not reported). Somatic cell counts tended ($P < 0.1$) to be greater following both ACTH and transport treatments than in controls (122 ± 23, 149 ± 25 and 66 ± 23 x10^3 cells ml^{-1}, respectively).

Discussion

Previous reports, in which cortisol concentrations in milk and plasma were demonstrated to be in a state of dynamic equilibrium, were based on experiments which used time frames extending from 4h after treatment up to several days (Gwazdauskas *et al* 1977; Bremel and Gangwer 1978; Fox *et al* 1981; Shutt and Fell 1985). The results of the present study reveal the nature of this equilibrium when activity of the HPA axis was manipulated during the 4h immediately before milking.

The magnitude and duration of the increases in plasma cortisol concentrations following treatment with ACTH, were similar in both experiments to those reported previously following intravenous injection of 0.05mg $ACTH_{1-24}$ into non-lactating dairy cows (Verkerk *et al* 1994). Thus, by varying the timing of saline or ACTH injection in Experiment 1, we were successful in producing a wide range of plasma cortisol concentrations at the time of afternoon milking (range, 3.8 – 76ng ml^{-1}). In Experiment 2, a range of plasma cortisol concentrations at milking time were engineered either by administration of ACTH, or by eliciting a stress response to trucking (range, 2.8 – 93ng ml^{-1}).

Our data suggest that measurement of composite milk cortisol concentrations as an indicator of exposure to stress may be limited to occasions in which samples are obtained during, or very shortly after, a period in which plasma cortisol concentrations are elevated. In both experiments, milk cortisol concentrations closely reflected plasma cortisol concentrations at milking, but not the integrated adrenocortical responses over the previous 4h. Measurement of concentrations of cortisol in milk samples failed to detect elevations in plasma cortisol concentrations which occurred more than 1h before milking. Mean cortisol concentrations for Group D, milked at about the time of peak plasma cortisol concentrations, were $56.0 \pm 6.5 ng ml^{-1}$ and $12.4 \pm 2.4 ng\ ml^{-1}$ in plasma and milk, respectively. By comparison, mean cortisol concentrations for Group C were $23.9 \pm 5.1 ng\ ml^{-1}$ and $2.4 \pm 0.8 ng\ ml^{-1}$ in plasma and composite milk, respectively, despite plasma cortisol concentrations having peaked at 51.6 ng ml^{-1} just 1h previously. Furthermore, there was no evidence of the previous adrenocortical stimulation when milk cortisol concentrations were measured in samples collected 3h after ACTH administration (Group B). An experiment using a low-dose ACTH test, in which milk and blood are serially sampled via indwelling catheters, may best reveal the rapidity of this very dynamic relationship.

Whereas cortisol concentrations in composite milk changed rapidly as plasma cortisol concentrations changed, the mechanisms of cortisol flux may have influenced foremilk concentrations more slowly. This is demonstrated in the ratio of cortisol concentrations in foremilk and composite milk. In groups A and D, the cortisol concentrations in foremilk were similar to those in composite samples (foremilk:composite milk ratios, 0.7 and 0.9, respectively). In contrast, concentrations in foremilk were 1.6 and 2.5 times those in composite milk in Groups B and C, respectively. Although the higher fat content of foremilk has been observed to play a role in partitioning progesterone in this fraction (Schiavo *et al* 1975), cortisol does not appear to be retained here (Gwazdauskas *et al* 1977). The observed uneven distribution might be influenced by the distribution of milk within the udder prior to milking. Foremilk comprises about 20 per cent of the total milk, and accumulates in the cistern between milkings. The remaining 80 per cent of milk is retained in the alveoli and not released until milk let-down (Dewhurst and Knight 1993). The differing ratios suggest that when plasma cortisol concentrations are decreasing following a period of elevation (as

in groups B and C), the cortisol diffuses from milk within the alveoli back into the blood. In contrast, the diffusion of cortisol out of milk already in the cistern may occur less quickly. The reason for this is not known, but could be related to the closer association of alveolar milk to the vasculature of the udder which would permit rapid exchange of free cortisol between blood and milk until milk let-down. This opportunity may not exist for substances in the foremilk within the cistern. The implication of this finding is that foremilk may be a better medium for measuring cortisol concentrations in order to assess the impact of stressors acting some time prior to milking. This requires further investigation.

Treatments did not influence any milk composition variable; but whereas ACTH injection was without effect, transport significantly reduced milk weight. Similar responses to physiological stressors have been reported previously (Bremel and Gangwer 1978), but the effects of ACTH on milk production are equivocal (no effect: Fox *et al* 1981; decrease: Bremel and Gangwer 1978). There were variations in milk composition between individual cows and between the days of treatment in Experiment 2. This is not unexpected because there was no pre-selection of animals for production, and because significant day to day variation in metabolite concentrations of pasture-fed dairy cows can occur which will influence milk composition (Kolver and Macmillan 1993). The increases in mean milk cortisol concentrations between the morning and afternoon milkings seen in the control cows in both experiments might be attributed to either the increased handling associated with the experiment, to heat stress or to the presence of flies (since the experiment was carried out during summer). This demonstrates the need to control for exogenous stressors other than those imposed by the experiment.

Individual responses were similar to both ACTH challenge and the stress imposed by transport. The detection of a main effect of individual cows on adrenocortical response, as measured by plasma cortisol concentrations at 60min, and of significant covariance between plasma cortisol concentrations at 0min and 60min are of interest; as are observations that one third of the variance was attributable to a consistency of response by individual cows, and that the rank correlation between the two treatments was significant. A consistent capacity for adrenocortical response to ACTH administration, allowing characterization of the high and low responding extremes within a population, has been reported in pigs (Hennessey *et al* 1988). Further studies of the porcine stress response have developed the concept of coping strategies in which sympathoadrenal, adrenocortical and behavioural responses are considered in an integrated manner for the classification of individual animals (Geers 1995). Our observations suggest that similar consistencies may exist within cattle populations. This has implications for future stress research, not least because consideration of such consistencies should be made during randomization procedures of experimental design.

In conclusion, milk cortisol concentrations were a sensitive measure of plasma cortisol concentrations immediately prior to milking. They failed to indicate elevations which had occurred within 3h of milk sampling, where plasma cortisol concentrations had returned to basal levels. Cortisol concentrations in composite samples were a more accurate indication of plasma cortisol concentrations at the time of milk sampling, but foremilk samples may provide a better indication of exposure to stress 1-2h before sampling.

Animal welfare implications

An improved understanding of changes in cortisol concentrations in milk relative to those in plasma, and of the consequent limitations in the use of milk cortisol measurements to assess response to acute stressors, has been described. Since milk sample collection is a less stressful procedure than that of blood sample collection, strategic use of milk sampling and improve the welfare of experimental animals where stress responses are being monitored. This work also has wider implications for animal welfare as it contributes to an understanding of the relationship between adrenocortical responses and stress in cattle.

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